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Short Article

Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos

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SUMMARY

We report on the establishment of a human embryonic stem cell (HESC) line from a preimplantation fragile X-affected embryo and demonstrate its value as an appropriate model to study developmentally regulated events that are involved in the pathogenesis of this disorder. Fragile X syndrome results from FMR1 gene inactivation due to a CGG expansion at the 5′UTR region of the gene. Early events in FMR1 silencing have not been fully characterized due to the lack of appropriate animal or cellular models. Here we show that, despite the presence of a full mutation, affected undifferentiated HESCs express FMR1 and are DNA unmethylated. However, epigenetic silencing by DNA methylation and histone modification occurs upon differentiation. Our unique cell system allows the dissection of the sequence by which these epigenetic changes are acquired and illustrates the importance of HESCs in unraveling developmentally regulated mechanisms associated with human genetic disorders.

INTRODUCTION

Fragile X syndrome (FRAXA) is the most common form of inherited mental retardation (Crawford et al., 2001). It is inherited as an X-linked trait and is caused by the absence of the fragile X mental retardation protein (FMRP) (O'Donnell and Warren, 2002). The vast majority of fragile X patients do not express the fragile X mental retardation 1 (FMR1) gene due to a dynamic mutation that involves a CGG triplet repeat expansion in the 5′ untranslated region of the gene (Nolin et al., 1996; Verkerk et al., 1991). The number of CGG repeats varies so that normal individuals have between 5 and 55 CGG repeats, while affected patients have more than 200 copies (full mutation) (O'Donnell and Warren, 2002). FMR1 silencing by the CGG expansion was shown to be mainly attributed to epigenetic regulated transcriptional silencing, although CGG expansion can also cause translational suppression of FMRP to various degrees (Feng et al., 1995).

Full expansion of the CGG repeat usually coincides with hypermethylation of the repeat region and its upstream flanking CpG-island-type promoter (Oberle et al., 1991). This DNA hypermethylation has been shown to be coupled with several other epigenetic modifications, including histone H3 and H4 tail deacetylation, histone H3-K9 methylation, and histone H3-K4 de-methylation (Coffee et al., 2002; Coffee et al., 1999; Pietrobono et al., 2005; Tabolacci et al., 2005). These epigenetic events correlate with nuclease sensitivity differences (Eberhart and Warren, 1996) and seem to be critical for the transition from active to inactive chromatin configuration. However, the timing and molecular mechanism by which these changes are acquired in affected individuals during embryonic development are not fully understood, due to the lack of appropriate animal or cellular models. The currently available FMR1 mice, for example, which are useful for the study of the clinical phenotype (The Dutch-Belgian Fragile X Consortium, 1994), are inadequate for investigating the mechanism by which FRAXA is caused through CGG expansion (Bontekoe et al., 1997, 2001; Brouwer et al., 2007; Lavedan et al., 1997, 1998; Peier and Nelson, 2002). Primary and transformed cell cultures obtained from rare individuals of normal phenotype with an unmethylated full mutation in the FMR1 gene have been used to show that CGG expansion per se does not block transcription (Pietrobono et al., 2005; Smeets et al., 1995). A different approach is to study the FMR1 inactivation in affected fetuses. Several reports documented the timing of FMR1 silencing by examining FMR1 expression and DNA methylation in chorion villus samples and embryonic tissues of affected fetuses (Sutcliffe et al., 1992; Willemsen et al., 2002). Nevertheless, the use of the above-mentioned cell systems is restricted

in its potential to investigate developmentally regulated events and to track early events in the inactivation process.

Human embryonic stem cells (HESCs) have great value in the study of early embryonic development, as they can recapitulate embryogenesis by expressing developmentally regulated genes and by activating molecular pathways as they occur in vivo (Dvash et al., 2004). As such, they can be used to analyze the effect of specific mutations on particular developmental events, thereby allowing new insights on developmental processes that are otherwise inaccessible for research (Dvash et al., 2006). A powerful approach to produce a human ES cell line with a given mutation would be to derive cells from a genetically affected embryo, identified by preimplantation genetic diagnosis (PGD) (Pickering et al., 2005; Verlinsky et al., 2005). In this study, we have used this strategy to establish a human ES cell line with a full CGG expansion at the FMR1 locus. This cell line allows us to follow in detail early events in the process of FMR1 inactivation and thus is applied for unraveling the role of early embryonic differentiation in fragile X syndrome.

RESULTS

HEFX HESCs Derivation and Characterization

The FRAXA-affected cell line, named HEFX, was established from a blastocyst obtained during a PGD, performed on a FRAXA carrier with a premutation of 170 CGG repeats. PGD analysis indicated a male embryo that had inherited the abnormal allele. The newly established cell line displays all characteristics typical of HESC. It can be propagated for a long period of time in culture (currently at passage 30) without losing its typical properties. HEFX HESCs express a panel of markers specific to undifferentiated cells, including NANOG, REX1, alkaline phosphatase, OCT4 (Figures 1A–1C), and the cell-surface marker Tra-1-60. HEFX cells show a typical transcription profile of HESC, as determined by cDNA microarray analysis (data not shown). In addition, these cells have a morphology characteristic to HESC (Figure 1D). Pluripotency of HEFX cells was confirmed in vitro by generating mature embryoid bodies (EBs) in suspension culture (Figure 1E) and in vivo by inducing teratomas when injected under the kidney capsule of immunodeficient mice. Histological examination of embedded sections of HEFX-derived teratomas indicates a wide developmental potential, evident by the presence of various cell types and structures (Figure 1F). Chromosome analysis by Giemsa staining, carried out on 20 metaphase spreads at passages 8 and 16, shows a normal XY human karyotype (Figure 1G). Haplotype analysis by PCR of HEFX cells confirmed the results obtained by single-cell PGD analysis (data not shown). In order to verify whether the maternal premutation expanded into a full mutation, we directly determined the number of CGG repeats within the FMR1 locus of HEFX cells by Southern blot analysis. The analysis revealed the presence of the genetic lesion that characterizes FRAXA-affected patients, having a variable number of tandem repeats within the range of 200 to more than 1000 copies (Figure 1H).

FMR1 Expression in HEFX HESCs and Their Differentiated Products

In FRAXA patients, the expansion of CGG tandem repeat to more than 200 copies results in epigenetic silencing of FMR1 expression. Accordingly, the presence of a full expansion in our cells predicts that the FMR1 gene will be silenced. Nevertheless, HEFX cells actively transcribe FMR1 RNA at least as in wild-type HESCs, as determined by RT-PCR (Figure 2A). Furthermore, FMR protein (FMRP) is expressed as evidenced by western blot analysis using an antibody against human FMRP (Figure 2B). Expression levels in HEFX HESCs are lower than in the WT. This reduction, which is not in correlation with mRNA level, may be explained by low translation efficiencies due to the presence of the CGG expansion, as shown by Feng et al. (1995). The expression of FMRP in HEFX cells was also illustrated by immunostaining (see Figure 2Ca), while lymphocytic cell lines of normal and FRAXA-affected males served as controls (Figures 2Cb and 2Cc, respectively).

Although Southern blot analysis of HEFX HESCs cells clearly shows a full expansion in the triplet repeats, we wished to directly illustrate the expression of FMR1 from full mutation alleles. By isolating single-cell-immortalized clones, we were able to show that clones having alleles at the full-mutation range (>200 CGG repeats; Figure 2D) express both FMR1 mRNA (demonstrated by real-time RT-PCR analysis [Figure 2E]) and its protein (demonstrated by western blot analysis [Figure 2F]). Based on these findings, we propose that mutant FMR1 is transcriptionally silenced in a developmentally regulated fashion. Thus, we were interested in analyzing changes in FMR1 expression in the course of differentiation. To study the relation between differentiation and FMR1 inactivation, HEFX HESCs were induced to differentiate in vivo by generating teratomas in immunodeficient mice. Immunostaining analysis demonstrated that FMRP, which is predominantly expressed in discrete cell structures in WT teratomas (Figures 3Aa–3Ac), is diminished in HEFX-derived teratomas (Figures 3Ad–3Ae). SOX2 immunostaining identified these cell structures as primitive neuroepithelium (Figure 3Af). As an attempt to isolate a more homogeneous population of differentiated cells, teratomas were dissociated and continuously propagated in culture (passage 10–16, see the Experimental Procedures). We could demonstrate a significant decrease in FMR1 transcription levels during differentiation of HEFX cells as compared to WT by qRT-PCR (20-fold decrease as compared to only 2.5-fold, respectively) (Figure 3B). This reduction in FMR1 transcription correlates with a marked decrease in FMRP levels (Figure 3C). Our results indicate that FMR1 inactivation in HEFX cells is differentiation dependent.

Epigenetic Status of the FMR1 Locus in HEFX HESCs and Their Differentiated Derivatives

Since FMR1 inactivation in FRAXA patients is usually accompanied by DNA hypermethylation of the promoter region of the gene (CpG-rich sequence), methylation status was analyzed in undifferentiated HEFX cells and in their differentiated cell derivatives. Using DNA bisulfite

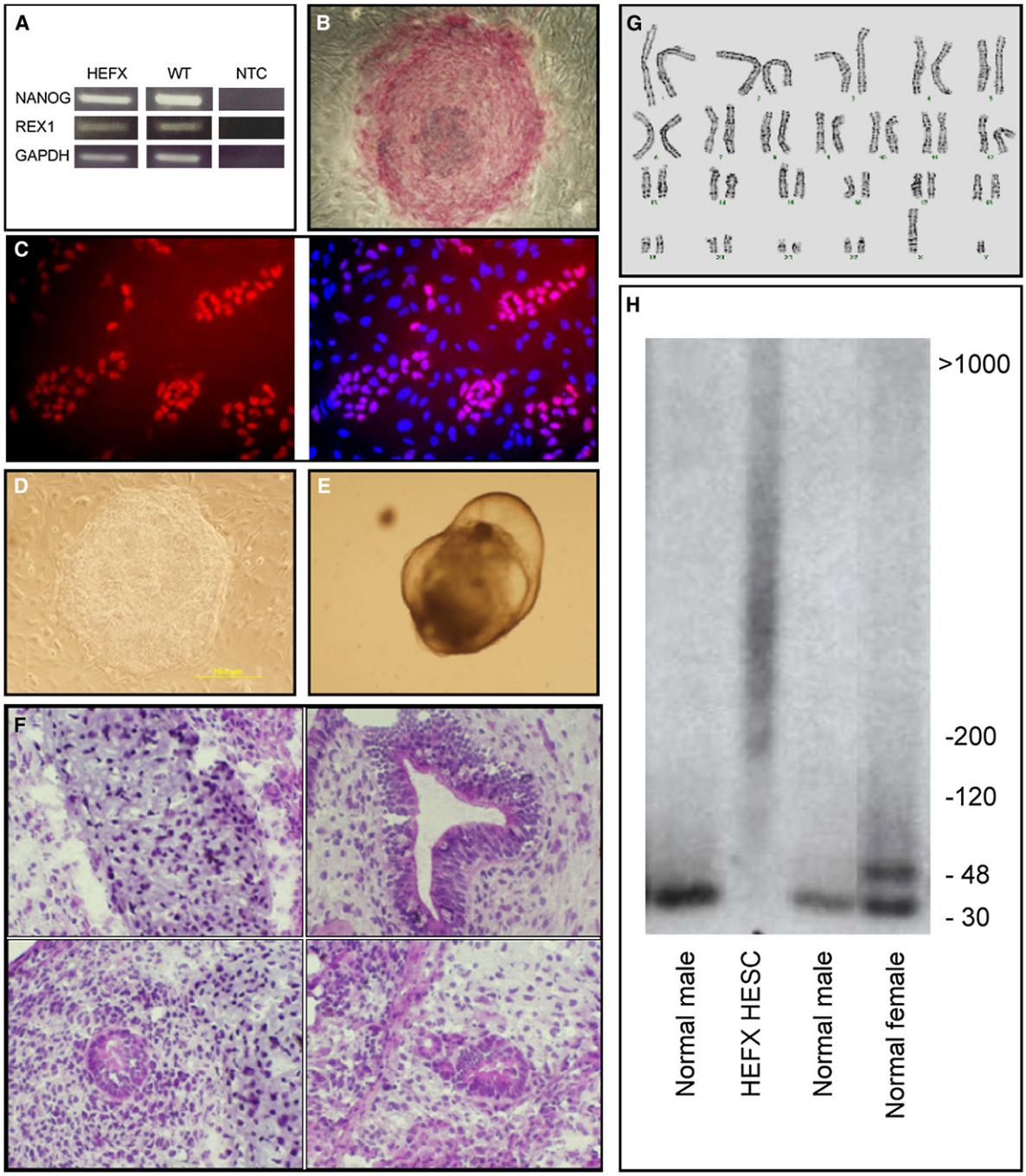

Figure 1. Characterization of HEFX Cells for the Expression of Undifferentiated Cell-Specific Markers and Pluripotent Potential

(A) RT-PCR products for NANOG, REX-1, and GAPDH using cDNA-specific primers. HEFX, fragile X-derived HESC line; WT, normal HESC line (HES13); NTC, no template control.

(B) Enzymatic staining for alkaline phosphatase of undifferentiated HEFX cells.

(C) Immunostaining for OCT4: staining for OCT4 and its overlay with Hoechst for nuclear staining.

(D) A colony of HEFX cells at passage 16 with a typical morphology of HESC.

(E) A cystic embryoid body (EB) established from HEFX cells grown for 20 days in suspension culture.

(F) Hematoxylin and eosin staining of 5-week-old HEFX-derived teratoma sections, showing various cell structures, including neural rosettes, cartilage, and glandular epithelium.

(G) Karyotype analysis for HEFX cells by Giemsa staining.

(H) Southern blot analysis for CGG repeat size by PstI digestion and hybridization with a radioactive ^{32}P-labeled probe, which detects a downstream flanking region to the CGG repeat. Normal alleles appear as discrete bands and range between 30 and 50 repeats (normal males and female) while fully expanded alleles range between 200 and 1000 repeats (HEFX cells).

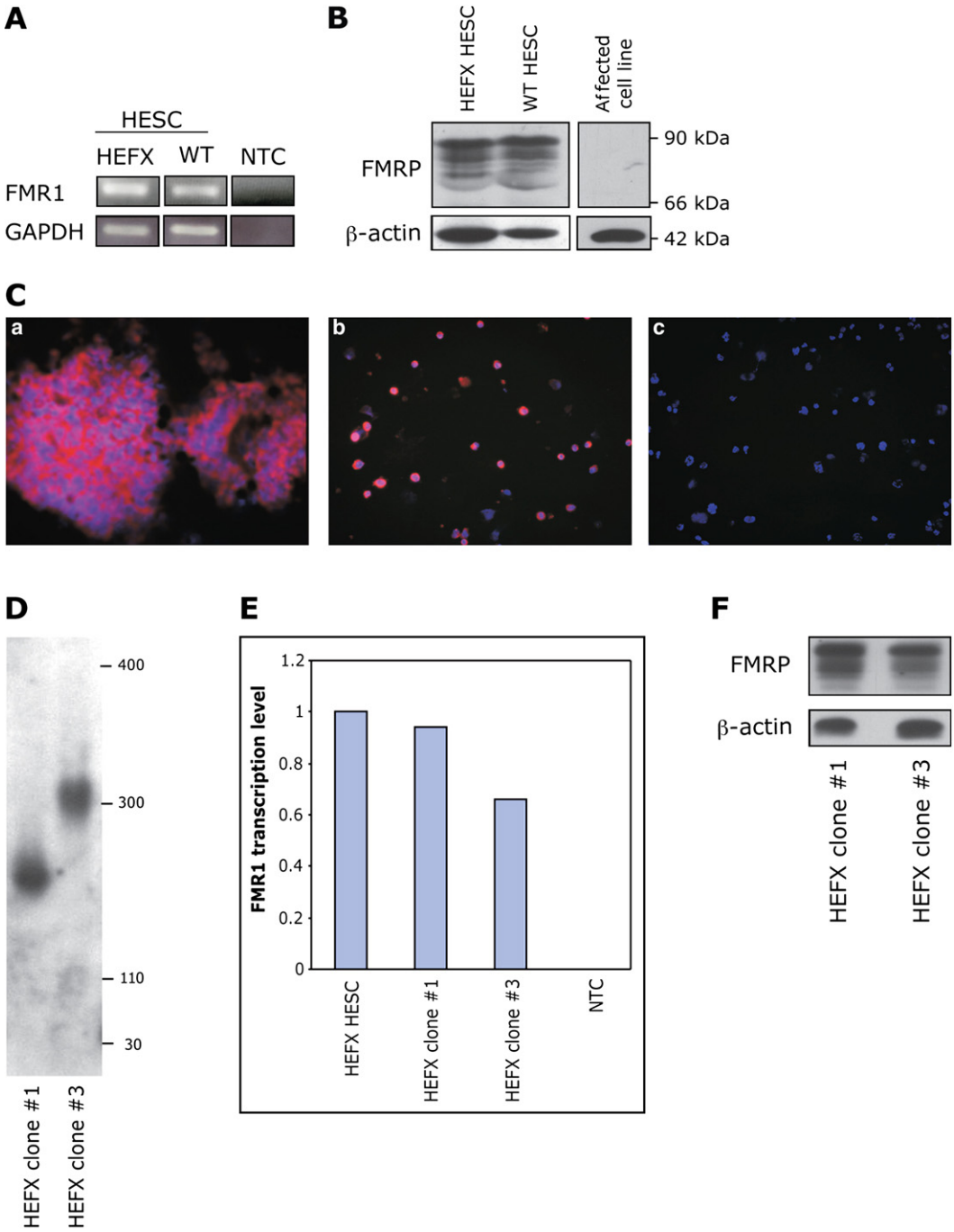

Figure 2. FMR1 Expression in Undifferentiated HESCs

(A) FMR1 RNA expression, detected by RT-PCR using cDNA specific primers in HESCs. HEFX, newly derived cell line from a male fragile X-affected embryo; WT, normal female HESC line (H9); NTC, no template control. GAPDH served as positive control.

(B) FMR1 protein expression, detected by western blot analysis using a monoclonal antibody against FMRP. WT, normal female ES cell line (H9); HEFX, newly derived cell line from a male fragile X-affected embryo; affected cell line, unexpressing lymphocytes cell line derived from a fragile X-affected male served as a negative control. β-actin served as a loading control.

(C) Immunostaining for FMRP in HEFX cells (Ca), WT lymphocysts (Cb), and fragile X-affected lymphocytes (Cc), using Cy3-conjugated mouse anti-human FMRP and Hoechst for nuclear staining.

(D) Southren blot analysis for CGG repeat size of two HEFX immortalized subclones (HEFX clone #1, HEFX clone #3).

(E) Real-time PCR analysis of FMR1 expression in HEFX HESC and the two subclones; NTC, no template control.

(F) FMR1 protein expression in the two subclones, detected by western blot analysis using a monoclonal antibody against FMRP.

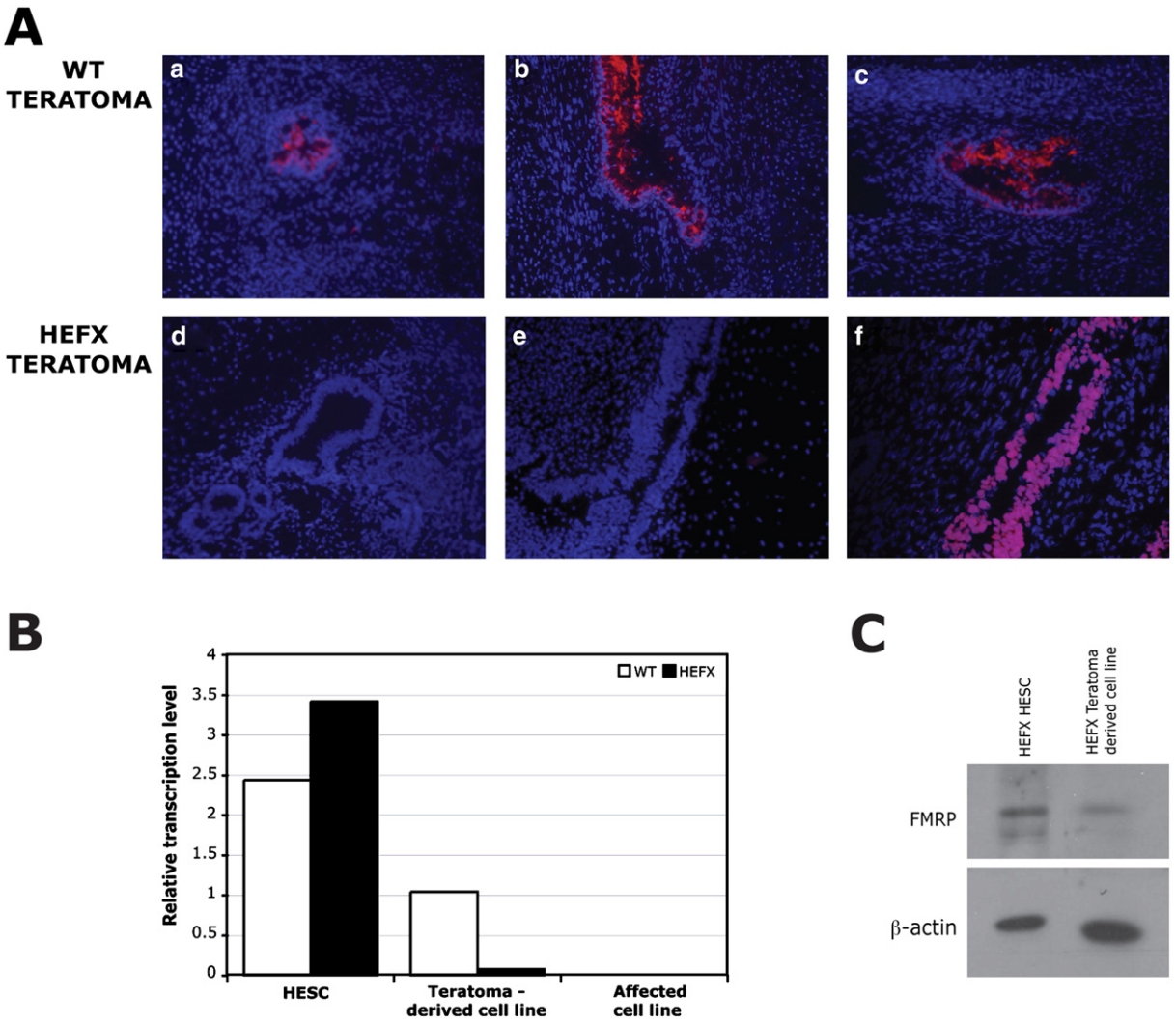

Figure 3. FMR1 Expression in Differentiated HEFX Cells

(A) FMRP expression in HEFX teratomas. Frozen sections were stained for FMRP in WT (Aa–Ac) and HEFX-derived teratomas (Ad and Ae). WT teratomas were also stained for SOX2 (Af).

(B and C) Downregulation of FMR1 expression in differentiated teratoma-derived cells, as determined by qRT-PCR (B) and western blot analysis (C). HESC, WT and HEFX undifferentiated ES cell lines. Affected cell line, unexpressing lymphocyte cell line derived from a fragile X-affected male served as a negative control.

treatment, followed by a methylation-specific PCR (MSP) assay, we show that the DNA at the FMR1 promoter of undifferentiated HEFX HESCs is unmethylated (Figure 4A, HESC). DNA methylation is acquired only upon differentiation, in EBs as well as in teratomas (Figure 4A, differentiation of HESC). In order to quantify the level of DNA methylation following differentiation, HEFX-derived tertomas were analyzed for methylation status by bisulfite sequencing of single genomic DNA clones. Although MSP PCR analysis demonstrated that DNA methylation is induced by differentiation, it occurs at very low levels in the teratomas (<5%; bisulfite sequencing). Nevertheless, since downregulation of FMR1 transcription takes place in the teratoma-derived cell line (Figure 3B), we were interested in looking at their chromatin composition. For this purpose, we have examined two specific histone modification markers that are associated with transcriptional activity and also have been coupled with FMR1 silencing in somatic cells of patients (Coffee et al., 1999, 2002). Histone H3 tail acetylation and histone H3 methylation at lysine 9 (H3K9 methylation) are two relatively early posttranslation modifications that correlate with positive and negative regulation of transcription, respectively. We thus carried out chromatin immunoprecipitation (ChIP) experiments to follow possible changes of these modifications in HEFX HESCs during differentiation. We have analyzed the 5′UTR region of FMR1 in HEFX HESCs as well as in the HEFX-derived teratoma cell line, where downregulation of

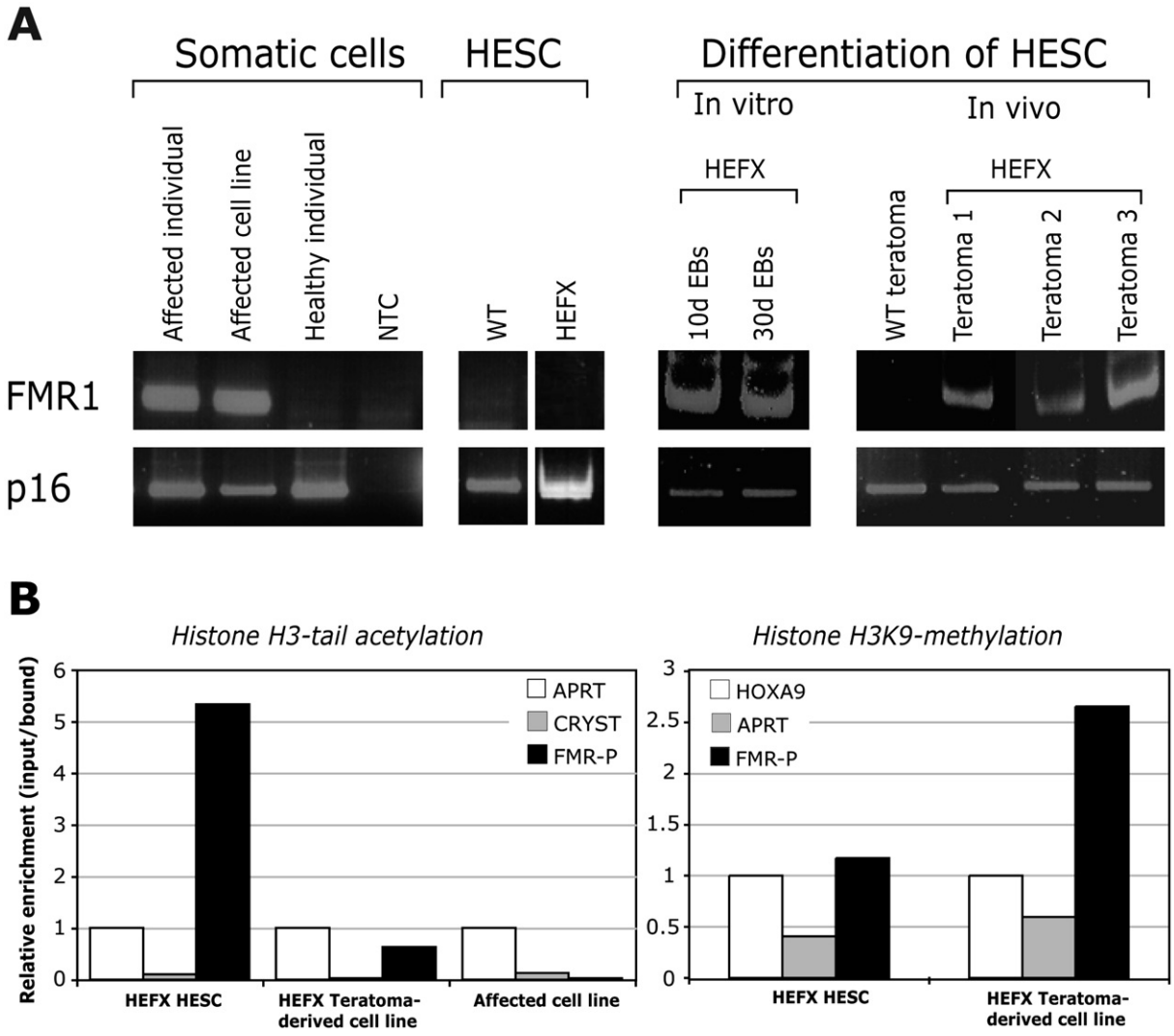

Figure 4. Epigenetic Modifications of the FMR1 Locus in HEFX HESCs and Their Differentiated Products

(A) DNA methylation status of the 5′UTR of FMR1. Differential amplification of methylated alleles at the 5′UTR of FMR1 was performed by PCR following bisulfite treatment of genomic DNA, as previously published by Panagopoulos et al. (1999). P16 amplification served as a positive control for bisulfite treatment using appropriate primers. Somatic cells: affected individual, lymphocytes of fragile X-affected individual; affected cell line, lymphocytes of fragile X-transformed cell line; healthy individual, WT lymphocytes from healthy individual; NTC, no template control. HESC: WT, normal undifferentiated male cells (HES13); HEFX, undifferentiated TRA-1-60-positive HEFX cells sorted by FACS (see the Experimental Procedures). Differentiation of HESC: in vitro, differentiation of HEFX cells into 10- and 30-day-old EBs. In vivo, differentiation of WT HESCs (HES13) and HEFX cells into teratomas.

(B) ChIP analysis of histone H3-tail acetylation and H3K9 methylation in HEFX cells. Real-time PCR was conducted on bound and input DNA fractions using primers for FMR1 promoter (FMR-P), APRT and crystaline (CRYST) as positive and negative controls for H3 acetylation, and HOXA9 and APRT as positive and negative controls for H3K9 methylation. Values were normalized relative to the appropriate positive control.

transcription was observed. These ChIP experiments show that, similar to normally FMR1-expressing somatic cells, the FMR1 promoter in undifferentiated HEFX cells is highly enriched in acetylated histone H3 and is unmethylated at H3K9 (Figure 4B). However, as a result of differentiation, histone H3 acetylation levels dramatically fall. The decrease in acetylated histones is coupled with methylation at H3K9. We thus conclude that FMR1 inactivation initiates by down-regulation of transcription and chromatin modifications prior to DNA methylation.

DISCUSSION

Studies related to human embryonic development, especially those associated with genetic disorders, are usually confined to animal models. Murine models, however, often fail to recapitulate the features observed in humans. For some genetic disorders, it is possible to genetically manipulate HESC to model the disease, as we had previously demonstrated for Lesch-Nyhan disease (Urbach et al., 2004). In the case of fragile X syndrome, the molecular

hallmark, namely the inactivation of FMR1 due to a CGG triplet repeat expansion, does not occur in the murine system (Brouwer et al., 2007). Here we used a newly derived HESC line (HEFX), which harbors a full CGG mutation at the FMR1 locus, in order to construct a valid research model. This model was used to study the timing and mechanism by which normal expression of FMR1 is inactivated during early embryonic development in embryos that carry the FMR1 full mutation. Using this cell line, we have shown that in the undifferentiated state the FMR1 gene is transcribed and translated. The normal levels of transcription are consistent with the results demonstrating that the promoter region is unmethylated and displays the typical features of active chromatin. These findings imply that the CGG expansion by itself is not sufficient to cause FMR1 inactivation, as also observed by Sutcliffe et al. (1992), who reported hypermethylation and lack of FMR1 transcription in embryonic tissues, but not in chorionic villi of a 13-week-old affected fetus. As an attempt to reset the epigenetic state of the FMR1 region into its embryonic state, cell reprogramming was induced in somatic cells of FRAXA patients by cell fusion (Burman et al., 1999; Wohrle et al., 2001). These studies have predicted a possible relationship between embryo development and FMR1 gene silencing in full mutation alleles. Embryonic stem cells, however, are undifferentiated cells in origin and therefore served as a better research model. Using this system, we showed, to our knowledge for the first time, that FMR1 inactivation is indeed triggered by differentiation.

FMR1 gene silencing is a multistep process that involves epigenetic changes in DNA and chromatin composition. Spontaneous differentiation of HEFX cells into EBs and teratomas induces DNA methylation, although to low levels. Given the heterogeneous-cell-type nature of teratomas and EBs, we aimed to examine chromatin state in a more homogeneous population of differentiated cells such as teratoma-derived cells. By growing these cells in culture for a long period of time, we could demonstrate a decrease in FMRP as well as in transcription activity of FMR1. This decline in mRNA levels is coupled with histone H3 deacethylation and histone H3K9 methylation, both epigenetic modifications that mark the DNA for heterochromatinization (Grewal and Jia, 2007). These changes occur despite the fact that the DNA is still hypomethylated at the 5′UTR of FMR1. These results somewhat differ from those of Pietrobono et al. (2005), who, based on the study of rare individuals, found that these two epigenetic marks that precede DNA methylation do not interfere with active gene transcription. This discrepancy emphasizes the great advantage of our system, which recapitulates early embryogenesis and thus enables us to identify intermediate forms in the cascade leading to the inactivation process, without relying on unusual molecular events. Identification of cells that reach a define stage during the process of inactivation might allow further dissection and perhaps better understanding of the molecular mechanisms that are responsible for FMR1 inactivation in FRAXA-affected embryos. Taking all these data together, we conclude that de novo methylation of the upstream regulatory region of FMR1 occurs following transcriptional inactivation and that the setting of specific chromatin modifications takes place relatively early during the course of differentiation. Our data also imply that DNA methylation of the FMR1 promoter contributes to the maintenance, rather than to the establishment, of the inactive state of the gene in somatic cells of patients. This is consistent with the possible role of DNA methylation as a locking mechanism from transcriptional reactivation, as was reported for CpG island genes on the inactive X chromosome in females (Lock et al., 1987) and for several embryonic genes (Feldman et al., 2006).

Given that HESCs can, to some extent, recapitulate early embryonic development, they can serve as an important tool to study developmentally regulated biological phenomena as they occur in utero (Dvash and Benvenisty, 2004; Eiges and Benvenisty, 2002). This is especially advantageous in cases where the biological phenomenon is tightly linked with differentiation, as in the case of FRAXA. It is currently unclear how CGG repeat expansion at the FMR1 locus leads to gene inactivation in affected fetuses. However, the fact that DNA methylation, histone modifications, and FMR1 inactivation take place only when differentiation is induced makes our system unique. It suggests that it might be possible to prevent the inactivation and methylation of the FMR1 gene, as an attempt to rescue the abnormal phenotype, in cells with full CGG expansion. The results of this study should advance our basic understanding in the evolvement of this important inherited disease and may have great value for medical applications in the future.

EXPERIMENTAL PROCEDURES

Preimplantation Genetic Diagnosis for Fragile X

A premutation female carrier with 170 CGG repeats copies at the FMR1 locus, who has a severely affected brother with more than 1000 CGG repeats, underwent IVF treatment for the purpose of PGD. At day 3 post fertilization, 12 embryos at the seven to eight cell stage were biopsied by the aspiration of two blastomeres from each and genetically analyzed for FRAXA as described (Malcov et al., 2007). PGD was carried out by multiplex PCR using the following markers: DXS998, CA1 and DXS1193, and the Sry gene. According to this analysis, five embryos were diagnosed as normal, while the other seven inherited the abnormal maternal allele.

HESC Derivation and Maintenance

The use of spare IVF-derived embryos that have been diagnosed as genetically affected by PGD for the generation of HESCs was approved by the Israeli National Ethics Committee (7/04-043).

FRAXA-affected embryos were cultured to the blastocyst stage. At day 6–7 of development, embryos were micromanipulated to remove the zona pellucida (ZP) and subjected to immunosurgery for the isolation of inner cell mass (ICM) cells, as described previously (Thomson et al., 1998). Isolated clumps of ICM cells were plated on MEF cells. Outgrowth-containing cells were manually cut and propagated, resulting in a stable culture of undifferentiated HESCs. Established cell cultures were grown as previously described (Eiges et al., 2001).

HESC Cell Differentiation

EBs

Undifferentiated cells were trypsinized and induced to form EBs by allowing them to aggregate in suspension culture by the growth in

nonadherent plastic petri bacterial dishes (Itskovitz-Eldor et al., 2000; Schuldiner et al., 2000) in the absence of bFGF. EBs were collected for analysis following 10 and 30 days of cell aggregation in culture.

Teratoma

All animal experiments were conducted under the supervision of the Hebrew University Faculty of Sciences Animal Care and Use Committee (license NS-01-05). Teratomas were formed by injection of 1–5 × 10^6 ES cells, under the kidney capsule of SCID/beige mice. Teratomas were isolated 5–8 weeks following injection. Histological sections were made by either paraffin embedding for pathological examination or fast cryopreservation for immunostaining. Pathological examination was performed by morphologically assessment of teratoma sections following hematoxylin and eosin staining.

Teratoma-Derived Cell Culture

Isolated teratoma was manually dissociated into small cell clumps and further trypsinized. The dissociated cells were than plated on gelatin-coated tissue culture plates and propagated in MEF medium.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted using TRI-reagent (Sigma), and 1 μg of RNA was reverse transcribed by random hexamer priming using Improm II First Strand cDNA Synthesis Kit (Promega). Amplification was performed on cDNA using Dynazyme Taq DNA polymerase (Finezyme) in the presence of X1 Taq Buffer, 200 μM dNTPs each, and 2.5 mM Mg^{2+}. A full description of primers, annealing temperature, and size of final products is described in Table S1, in the Supplemental Data available with this article online. Final products were assessed by gel electrophoresis on 2% agarose ethidium bromide-stained gels.

Real-Time PCR Analysis

Real-time PCR, carried out in three duplicates using SYBR Green ROX Mix (ABgene), has been done using 7300 Real-Time PCR System (Applied Biosystems). The RPL13A was used as an endogenous control. A full description of primers, annealing temperature, and size of final products is described in Table S2.

Staining of Cells for Expression of OCT4 and Alkaline Phosphatase

Antibody staining for *OCT4* expression was performed as previously described (Darr et al., 2006) by using monoclonal mouse Oct4 (Santa Cruz Biotechnology, Inc.; dilution 1:100) and Cy3-conjugated goat anti-mouse polyclonal antibodies (Jackson ImmunoResearch, Inc.; 1:200). Nuclear staining was performed with Hoechst 33258 (Sigma, Inc.). Staining for alkaline phosphatase was carried out using Alkaline Phosphatase kit (Sigma Diagostics, Inc.), according to the manufacturer's protocol.

Immunostaining for FMR1 and SOX2 Expression

Teratoma sections were fixed in cold methanol, blocked, and stained with mouse anti FMRP monoclonal antibody (Chemicon International, MAB2160, 1:100) or goat anti human SOX2 polyclonal antibody (Santa Cruz sc-17320, 1:50), using Cy3-conjugated as a secondary antibody.

HESC Sorting by FACS

FACS analysis for the undifferentiated ES cells was performed as described using TRA-1-60 antibody (kind gift from Prof. Peter Andrews). Samples were analyzed using the FACSCalibur system (Becton Dickinson) with CELLQUEST software (Becton Dickinson).

Southern Blot

PstI-digested genomic DNA was size separated by electrophoresis on a 1.2% agarose gel. Enzyme-restricted DNA samples were blotted onto Hybond N^+ (Amersham) and hybridized to a PCR-generated probe, using primers F, 5′GCTAGCAGGGCTGAAGAGAA, and R, 5′CAGTGGAGCTCTCCGAAGTC (595 bp long), which detects a tightly linked downstream sequence to the CGG repeats.

Western Blot Analysis

Western blot analysis was carried out by the use of monoclonal antibody against human FMR1 protein (Chemicon, 1:100 dilution) and a peroxidase-conjugated rabbit anti-mouse antibody (Jackson Immunoresearch, 1:10,000 dilution). Mouse anti-β-actin antibody (Abcam, 1:25,000 dilution) served as a loading control.

Methylation-Specific PCR

Genomic DNA was modified by bisulfite treatment using EZ DNA Methylation Kit (Zymo Research) and analyzed by PCR using FastStart DNA Polymerase and primers designed to specifically detect methylated alleles, as described by Panagopoulos et al. (1999). Universal primers for both methylated and unmethylated alleles were used for the amplification of p16 gene as an internal control (see Table S3 for primer sequence and PCR conditions).

Bisulfite Sequencing

Bisulphate sequencing was applied to determine the methylation status of 53 potential methylation sites at the promoter region of FMR1, which are known to be extensively methylated in somatic cells of affected individuals (Pietrobono et al., 2002). Genomic DNA was modified by bisulfite treatment and amplified by FastStart DNA Polymerase using primers designed to avoid any CpGs in their sequence (Table S2). Amplified products were cloned into Topo II TA cloning vector (Invitrogene). Single colonies were analyzed for CpG methylation at all potential sites by direct sequencing.

Isolation of Immortalized Single-Cell-Derived Clones by Retroviral Infection

HEFX undifferentiated cells, grown on matrigel in the presence of MEFs-conditioned media, were infected with ecotropic receptor, pBabe-hTERT-hygro, and ψ2SV40(T-Ag)-puro retroviral constructs. Infections were carried out in the presence of 8 μg/ml polybrene (Sigma) for 3 hr and then for 12 hr the day after. Immortalized single-cell-derived clones were isolated following a 5–10 day selection with 1 μg/ml puromycin and 140 μg/ml hygromycin.

ChIP Analysis

ChIP was performed according to previously published protocols with small modifications. In short, cells were crosslinked with formaldehyde solution, lysed, and sonicated to shear DNA to small fragments of 200–1000 bp long. Chromatin was then extracted and immunoprecipitated using salmon sperm agarose beads (Upstate Biotechnology) and antibodies directed against either acetylated histone H3 (K9, K14) (5 μg per 30 μg DNA) or 2.3 meth-H3(K9) (15 μg per 10 μg DNA). Crosslinking was reversed and DNA was recovered using QIAGEN Clean-Up Kit. Bound DNA was quantitatively compared to a 1:100 dilution of the input DNA. To amplify DNA molecules from bound and input fractions, real-time PCR was conducted using primers for FMR1 promoter region as well as for the appropriate positive and negative controls (APRT, CRYSTALIN, HOXA9, see Table S4).

Supplemental Data

Supplemental Data include four tables and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/5/568/DC1/.

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